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(54) Title: VISUALIZATION OF ENZYME-CATALYSED REACTIONS USING PH INDICATORS: RAPID SCREENING OF HYDROLASE LIBRARIES FOR ENANTIOSELECTIVE ENZYMES			
(57) Abstract <p>The use of pH indicators to monitor enzyme-catalyzed reactions is described. The formation of acid following an enzyme-mediated reaction, such as hydrolysis, causes a drop in the pH that can be visualized by a change in the color of the indicator-containing solution. Preferred indicators are those showing a color transition within the operational pH range of the enzyme. Using the present system, the enantioselectivity of enzymes such as lipases and esterases can be estimated using single isomers under the same conditions and comparing the color turnover for each one. The method is also useful for application to the hierarchical screening of a library of enzymes.</p>			

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***Visualization of Enzyme-catalyzed Reactions Using pH Indicators: Rapid Screening of
Hydrolase Libraries for Enantioselective Enzymes***

RELATED APPLICATIONS

This application is a claims priority to U.S. Ser. No. 60/091,880 filed July 7, 1998 and U.S. Ser. No. 60/125,708 filed March 23, 1999, both of which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

This invention relates to pH-dependent assays for detection of enzymatic activity.

BACKGROUND OF THE INVENTION

There is an increasingly important need to develop new biocatalysis processes rapidly and inexpensively, especially for the development of novel pharmaceuticals where time is extremely valuable. The use of a powerful, analytical screening strategy is often the key to speeding up development time at several different levels of the process. In the discovery of novel enzymes, screening plays an important role in identifying which subset of candidates contain an enzyme of interest from a collection of organisms, clone banks, or enzyme libraries. Directed evolution approaches to engineer custom biocatalysts require powerful screening strategies to sift through large mutant pools to find enzymes with properties that have often been only slightly altered against a high activity background. Finally, process optimization and development can often take an excessive amount of time, especially to perform a comprehensive analysis of different reaction conditions including temperature, pH, cosolvent, reaction time, and other parameters, both individually and in combination. This type of analysis requires the implementation of a rapid, high-throughput assay which is amenable to automation and use in a hierarchical screening strategy.

The synthesis of enantiomerically pure compounds (EPC) with one or several chiral centers is one of the most challenging tasks in modern organic chemistry. Enzymes are able to contribute significantly to this challenge and have been increasingly considered as a useful class of catalysts for organic synthesis. (Davies, G. et al. *Biotransformations in Preparative Organic Chemistry*; Academic Press: London, 1989; Wong, et al. *Enzymes in Synthetic Organic Chemistry*; Pergamon: Oxford, 1994; Faber, K. *Biotransformations in Preparative Organic Chemistry*, Springer-Verlag: Berlin-Heidelberg, 1995; Drauz, K; Waldmann, H Eds. *Enzyme Catalysis in Organic Synthesis Vol 1 & 2* VCH: Weinheim, 1995). Among these

biocatalysts, hydrolases are well established as valuable tools for the food, pharmaceuticals and fine chemicals industry. (Gerhartz W. Ed. *Enzymes in Industry* VCH: Weinheim, 1990). The importance of biocatalysis has led to the search of novel enzymes with singular activities. Recently, extremophilic microorganisms have been investigated as a source of these novel activities. (Kristjansson, J. K. *TIBTECH* 1989, 7, 349; Adams, et al. *Bio/Technology*, 1995, 13, 662; Govardhan, et al. *Chem. Ind.* 1995, 17, 689-93; Newell, J., *Chemistry in Britain* 1995, 31; Vieille, et al. *TIBTECH*, 1996, 14, 183).

Scientists at ThermoGen, Inc. (Chicago, IL) have developed a set of tools to obtain libraries of thermophilic enzymes by genetic engineering (see, for example, U.S. Pat. Ser. No. 08/694,078). ThermoCat® consists of a set of twenty stable esterases, capable of working well either at room or high temperature and also in organic solvents. It is of high interest to study the selectivity of each enzyme in the library, especially their enantiodiscrimination when exposed to racemic substrates. Time is the limiting factor in carrying out the work when screening a library of enzymatic activities against an array of substrates for either enzyme discovery, enzyme engineering (such as directed evolution) or process optimization experiments. The analytical methods typically employed for this purpose include high-pressure liquid chromatography (HPLC), thin-layer chromatography (TLC), and gas chromatography (GC), which are often not amenable to high-throughput assays.

The need in the industry for new methods for the identification of new biocatalysts requires rapid screening assays combined with hierarchical screening strategies. The approach works by eliminating the weakest candidates as one of the earliest steps in the bioprocess development timeline, rendering a streamlined process-viability study. (See, for example, Demirjian, et al. *Top. Curr. Chem.*, 1998, 200.)

One of the most convenient ways to assay an enzyme is through a method that allows the development of color and thus can be used in qualitative as well as quantitative measurements. A number of colorimetric methods to measure enzymatic activity have been described. (Michal, et al. in *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Verlag Chemie: Weinheim, 1983; Vol. I; pp 197; Demirjian, et al. *Top. Curr. Chem.*, 1998, 200.) Hydrolytic enzymes can be rapidly screened with chromogenic (nitrophenyl), fluorogenic (4-methylumbelliferone) or indigogenic (indoxyl) substrates that yield colored products upon hydrolysis. The main limitation of this approach is the presence of the latent colorimetric functionality within the substrate, whose introduction is at least time-consuming and yields a structure for analysis essentially different from the actual target (usually a methyl or ethyl ester).

Several types of pH-dependent assays have been described including enzyme-catalyzed processes with hexokinase. (Wajzer, J. *Hebd. Seances Acad. Sci.*, **1949**, 229, 1270; Darrow, et al. *Methods in Enzymology*, **1962**; Vol. V, 226; Crane, et al. *Methods in Enzymology*, **1960**; Vol. I, 277) and cholinesterase (Lowry, et al. *J. Biol. Chem.* **1954**, 207, 19) and in enzyme-free studies of carbon dioxide hydration. (Gibbons, et al. *J. Biol. Chem.* **1963**, 238, 3502). Since the 1970's, such strategies have been used in kinetic analysis of enzyme reactions. Examples of this include human carbonic anhydrase (Khallifah, R. G. *J. Biol. Chem.*, **1971**, 246, 2561), amino acid decarboxylases (Rosenberg, et al. *Anal. Biochem.* **1989**, 181, 59) and serine proteases (Whittaker, et al. *Anal. Biochem.* **1994**, 220, 238). The progress of the hydrolysis is monitored by visual inspection of the solution color after the enzyme has been added or by using a microplate reader to get a quantitative reading.

Whittaker et al. (*Anal. Biochem.* **1994**, 220, 238-243) measured the esterase activity of proteases in 96-well microplates using a pH-dependent assay. However, the Whittaker assay requires additional experiments beyond those required in practicing the methodologies provided herein in that Whittaker does not use an indicator-buffer pair with the same pK_a values and does not reliably measure the true rates of enzyme-catalyzed hydrolysis.

Recently, the use of pH indicators has been extended to monitor the directed evolution of an esterase on a plate assay using a whole cell system, rather than the isolated enzyme. (Bornscheuer, et al. *Biotechnol. Bioeng.* **1998**, 58, 554). However, a pH-dependent assay has not been utilized to determine the enantioselectivity of an enzyme. Kazlauskas has partially solved the problem of competition by using a reference non-chiral additive in classical chromogenic substrate assays. (Janes, et al. *J. Org. Chem.*, **1997**, 62, 4560.) The same author developed a quantitative method for the evaluation of the enantioselectivity (without considering the competition factor) for actual substrates based on a pH indicator/buffer system (*p*-nitrophenol / BES) with equal pK_a so the linearity of the color transition allows the quantitation of the enantioselectivity. (Janes, et al. *Chem. Eur. J.*, **1998**, 4, 2317).

Despite its impressive accuracy and sensitivity, the method of Kazlauskas requires special instrumentation (i.e., a microplate reader) since the color transition cannot be visualized and involves data management that could be avoided by using a suitable indicator that effectively turns color so the monitoring could be simplified. Furthermore, in certain cases, the linearity of the assay (and consequently its accuracy) is compromised by the difficulty of choosing a pair buffer / indicator with same pK_a and yielding color change.

As a solution to the deficiencies in the currently available art, provided herein is a simple, colorimetric, pH responsive method for the rapid screening of enzyme libraries is provided herein. This is based on the pH change (typically to a lower pH) that occurs as the reaction proceeds and the carboxylic acid is released. This drop can be monitored by visibly detectable change in color of a solution as determined by a pH indicator, where the color profile of the indicator falls into the pH range of the enzymatic activity. Using this methodology, the reactivity (detected by a color change) of pairs of enantiomers corresponding to the same racemic mixture can be detected and an estimate of the enantioselectivity of the enzyme can be made. A large turnover difference between isomers indicates a high probability of successful kinetic resolution if the racemic mixture is subjected to the enzyme displaying such time difference. The method involves the use of single isomers, so the kinetics obtained in this way do not reflect the competition that exists when hydrolyzing the racemic mixture, and therefore E value is approximate, but very effective, especially in the case high E, which are the important ones when screening libraries of enzymes and substrates.

SUMMARY OF THE INVENTION

Provided herein is a method for the visualization of hydrolase-catalyzed reactions using pH indicators by combining an enzyme to be tested, a substrate to be tested, an appropriate buffer, and a pH-sensitive indicator in a reaction mixture where the indicator is selected based on the predicted chemical reaction of the enzyme and the substrate. In one embodiment, the indicator will change the color of the reaction mixture in relation to progress of a chemical reaction between said enzyme and said substrate. The assay is useful for determining qualitatively the enantioselectivity or stereoselectivity of an enzyme against pairs of enantiomers or stereoisomers. In a preferred embodiment, the assay is useful for determining the enantioselectivity of an enzyme. In another embodiment, the assay is useful for the identification of DNA molecules encoding ester-hydrolyzing enzymes. In yet another embodiment, the assay is useful for detection a mutation to a DNA molecule encoding an ester-hydrolyzing enzyme that alters the activity of the enzyme.

BRIEF DESCRIPTION OF THE FIGURES

- Figure 1.** pH-dependent assay using a) bromothymol blue; and, b) phenol red.
Figure 2. Hierarchical screening of a library of thermophilic esterases.

DETAILED DESCRIPTION

While twenty enzymes were until recently considered a large number to screen in the development of a new biocatalytic process, it is only a modest number by today's standards. Increasingly, enzyme libraries are becoming larger and larger, and an effective tool for screening and comparison is becoming a necessity. Provided herein is a pH-dependent methodology for quickly and efficiently optimizing reaction parameters (such as conditions for the stereoselective transformation of a molecule) for identification of enzymes having enantiomeric selectivity. In this manner, only a small subset of reactions need to be analyzed in more detail, such as by HPLC. This will significantly increase the number of samples and conditions that can be analyzed in a short period of time.

In one embodiment, the assay provided herein is useful for qualitatively determining the selectivity of an enzyme, including but not limited to the enantioselectivity or stereoselectivity of an enzyme, against pairs of enantiomers or stereoisomers. The assay is useful to identify enzymes having activity towards compounds with more than one chiral center, such as diastereomers. In addition, the assay is also useful for the study of enantiomeric mixtures where chirality is determined by symmetry axis or rotational barriers.

Reagents and methodologies for the use of pH indicators to monitor hydrolase-catalyzed reactions are provided herein. In one embodiment, the formation of acid following an enzyme-mediated hydrolysis causes a drop in the pH that may be visualized by a change in the color of the indicator-containing solution. Preferred indicators are those showing a color transition within the operational pH range of the hydrolases, including but not limited to bromothymol blue and phenol red. The enantioselectivity of lipases and esterases may be estimated using single isomers under identical conditions and comparing the color turnover for each one. In one embodiment, this method may be utilized to quickly evaluate the enantioselectivity of a lipase towards a set of ester substrates and applied to the hierarchical screening of a library of thermophilic esterases.

Several advantages are provided by the methods provided herein. For example, as the signal being monitored (ie, color change) does not originate from the substrate but from the indicator, the actual substrate can be used for screening. Currently, custom-made fluorogenic or chromogenic substrates are utilized but the reactions may vary because the substrate has been altered. Another advantage is that the enantioselectivity of the enzyme can be evaluated if both enantiomers of the racemic mixture are tested separately, typically suboptimal but adequate for the qualitative screen demonstrated herein. Also, if both enantiomers are not

available, the comparison of the results obtained using the racemic mixture compared to the results obtained using the available enantiomer will provide useful data. In that case, the available enantiomer has to be the slow-reacting isomer of the racemic mixture. Yet another advantage is that the present assay does not require complicated hardware devices, and is based only on visual observation of the color change of the reaction mixture. And, since the assay is very sensitive, very small amounts of substrate relative to enzyme are required to detect the color change, opening the use of microplate wells and the subsequent automation of liquid handling systems.

Any suitable buffer may be utilized in practicing the present invention. Many such buffers are known in the art. For instance, the use of phosphate buffer at a pH equal to its pK_a (7.20) (Beynon, et al. in *Buffer Solutions: The Basics*, IRL Press, Oxford, 1996) would be preferred because it is compatible with the function of the majority of lipases and esterases and their stability profile. The phosphate buffer also provides mild conditions (neutral pH) if sensitive substrates are to be hydrolyzed. It is also standard to utilize a pH slightly above or below neutral if the enzyme selected shows more desirable activity under these conditions. Suitable buffers may include, but are not limited to inorganic buffers such as potassium phosphate (pH 6-8), sodium phosphate (pH 6-8), or sodium carbonate (pH 8-10); or organic buffers such as BES (N,N-bis[2-hydroxyethyl]-2-aminomethanesulfonic acid, pH 6-8), Tris (pH 7-9), citric acid (pH 4-6), and HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid; pH 6-8).

The use of an indicator dye with which to follow the reaction forms another part of the present invention. Exemplary pH indicator dyes suitable for following the hydrolytic reaction by the color change in the range typically used for this class of biotransformations is shown in Table 1. Other indicators are known in the art, such as those described in Bányai, et al. (*Indicators*; Bishop, E., Ed.; Pergamon; Oxford, 1971, pp 65-176.) It should be understood that many such indicator dyes are available, and that any of those available dyes not listed in Table 1 may also be useful in practicing the present invention.

Table 1***Exemplary pH Indicators***

Name	pK _a	pH range	color change
Bromothymol Blue	7.30	6.0-7.6	yellow-blue
Neutral Red	7.40	6.8-8.0	red-amber
Phenol Red	8.00	6.6-8.0	yellow-red
Brilliant Yellow	-	6.6-7.8	yellow-red
Cresol Red	8.46	7.0-8.8	yellow-red
Turmaric (curcumin)	-	7.4-8.6	yellow-red
Metacresol Purple	8.3	7.4-9.0	yellow-purple
2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulphonic acid, disodium salt	-	6.0-7.0	yellow-blue
6,8-dinitro-2,4-(1H)quinazolinedione	-	6.4-8.0	colorless-yellow

For example, the bromothymol blue / potassium phosphate system falls within 0.1 units of pK_a (making it suitable for quantitation) and provides a useful blue-yellow color transition in a buffer commonly included in hydrolase-catalyzed biotransformations. In a preferred embodiment, Bromothymol Blue (BTB) or Phenol Red are utilized in combination with phosphate buffer, which has a similar pK_a value. In this manner, the color transitions show high contrast as opposed to using the phosphate buffer in combination with a dye such as Neutral Red, which has a similar pK_a but displays poor distinction between red and amber. It is to be understood by the skilled artisan that various operating pH conditions may be utilized based on the individual enzyme characteristics or process advantages, and that a different indicator may be utilized that corresponds to the operating pH conditions. For instance, in one embodiment, phenol red may be the optimal indicator where the reaction is carried out at slightly basic pH (i.e., pH 8.0). In a preferred embodiment, preliminary experiments are performed to determine the concentration of indicator dye in the reaction that has no effect on the reaction rate, suggesting that the indicator does not inhibit enzyme activity. In yet another embodiment, control experiments including a protein source such as BSA, for example, may be performed to demonstrate that the presence of protein does not alter the indicator color. In this manner, it can be confirmed that the pH changes in the solution were the result of enzyme catalyzed hydrolysis. Further tests of reaction solutions containing enzymes and indicators without substrates (control assays) may also be performed

to establish that the color changes in solution do not result from the presence of either the buffer salts or the enzymes without regard to their activity.

In one embodiment, the enantioselectivity of a biocatalyst may be monitored using an array of enantiomeric substrates described in the literature to be efficiently resolved by well-known enzymes. One such lipase biocatalysts is from *Pseudomonas cepacia* (PSL; available from Amano Pharmaceutical, Japan). PSL has been used both for enantioselective hydrolysis as well as transesterifications in organic solvents. Exemplary substrates that display enantioselectivities in the desired range (from high to very high) are shown in **Example 2**. These allow for the development of a successful biocatalytic resolution in a cost-effective manner. **Example 2** illustrates substrates ready to use in the hydrolytic assay. Certain substrates will be available only as alcohols (both isomers); such substrates may be acetylated prior to the assay.

Enantioselectivity (E) is related to the time it takes each enantiomer to change the color. The larger the difference between the times to color change for each enantiomer, the higher the enantioselectivity of the enzyme for the fast-reacting enantiomer. The resultant measurement is an estimated or relative enantioselectivity. Preferred enzymes demonstrate large enantioselectivity values (ie, fast time to color change), and would be understood to have the most potential for practical application.

In one embodiment, a reaction is set up in a reaction vessel, such as a 96-well microplate, and the reaction solution contains an enzyme showing hydrolytic activity such as a hydrolase (such as an esterase, lipases, and proteases), a substrate solution, a buffer solution, and an indicator dye. In a preferred embodiment, the enzyme is a lipase such as PSL and the buffer solution has a pH of 7.2. In a more preferred embodiment, the indicator dye is present at a concentration by volume of 0.1%, 0.01% or 0.001%. The amount of substrate will vary but may be in the range of 1 to 20 mg/ml final concentration in the reaction mixture, and is preferably about 10 mg/ml. This low concentration allows for the use of small amounts of pure enantiomer, which is often available in limited quantities. To optimize the assay, an amount of enzyme is selected that allows for a convenient time-window of preferably less than 48 hours for the turnover of one of the isomers. If the reaction is too slow with the selected amount of enzyme, the skilled artisan may choose to increase the amount of enzyme utilized or to raise the temperature of the reaction such that evaporation resulting in a dried reaction does not occur.

The concentration of the buffer to be employed may be determined empirically, but should be weak enough to be saturated by the carboxylic acid being produced and drive the

pH to the turnover point of the indicator. In a preferred embodiment, the concentration of the buffer is between 5 mM and 10 mM KPi buffers, which turn color faster, since their buffer strength is lower. In a more preferred embodiment, the concentration of the buffer is 20 mM. In another preferred embodiment, the buffer is 20 mM potassium phosphate at pH 7.2. Use of a buffer at too low a concentration (ie, less than 5 mM) may result in increased sensitivity of the system where minor pH shifts due to the background (after adding the enzyme or the substrate) may lead to inconsistent readings. In that event, correction of the pH in each distorted well may be required.

In practicing the present invention, the enantioselectivity corresponds with the faster turnover (development of a color change in the reaction mixture) of the preferred isomer. It is well within the skills of the ordinary practitioner to determine the appropriate time frames for use with certain reactions. If the enzymatic reaction proceeds slowly, a greater amount of time is required to monitor the progress of the reaction. In certain cases, the same biocatalyst may be utilized to evaluate a library of substrates, and the nature of the substrates will dictate the time frame of the assay. Although the actual biocatalytic transformation may occur under different conditions, such as different stirring conditions, the method provided herein generates estimates of initial reaction rates. This may serve to indicate the proper enzyme:substrate ratio required for the biotransformation of one substrate versus another. For cases in which only one of the isomers is available, the method functions most efficiently if the available isomer is the slower reacting one. Otherwise, both wells may change color almost simultaneously and no conclusion can be obtained from the experiment. As would be understood by the skilled artisan, it is not necessary for both isomers to run on a single screen.

In a preferred embodiment, a commercial kit including multiple enzymes of a particular class, such as a lipase, protease or esterase, may be organized into a microplate array in a buffer containing BTB, for example. In a preferred embodiment, the buffer has a Kpi of approximately 20 mM at pH 7.2. A first round of hierarchical screening is accomplished using all the enzymes in the kit to identify those enzymes reacting with the racemic substrate. At this point, the enzymes having the fastest reaction times are selected for further analysis. This first round prevents the unnecessary use of the oftentimes scarce pure isomers to screen out negatives.

The following Examples are for illustrative purposes only and are not intended, nor should they be construed as limiting the invention in any manner. Those skilled in the art

will appreciate that variations and modifications can be made without violating the spirit or scope of the invention.

EXAMPLES

Example 1

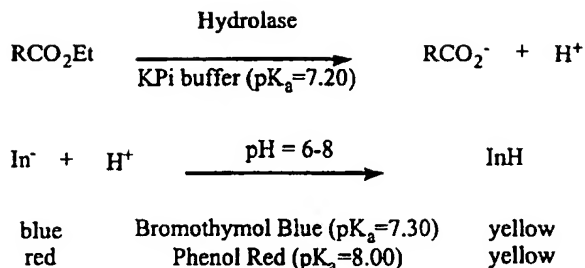
Materials and Methods

The enzymes used were obtained from Amano Pharmaceutical (Japan) and ThermoGen, Inc. (Chicago, USA). PSL was dissolved in buffer (10 mg/ml) and centrifuged before use to remove insoluble material. Both single isomers (R and S) of methyl mandelate, 1-indanol, 1-phenylethanol, and α -hydroxy- γ -butyrolactone were purchased from Aldrich (Milwaukee, USA) together with R-mandelonitrile acetate (R-5) and its corresponding racemic mixture. These were derivatized by acetylation to obtain both isomers of 1, 3, 4 and 6 together with the racemic 5. R- and S-ethyl 3-hydroxy-3-phenylpropionate (2) were purchased from Fluka (Switzerland) and used as is. All of the isomers were stocked in MeCN at 60 mg/ml. The phosphate salts and the indicators bromothymol blue and phenol red were obtained from Aldrich (Milwaukee, USA).

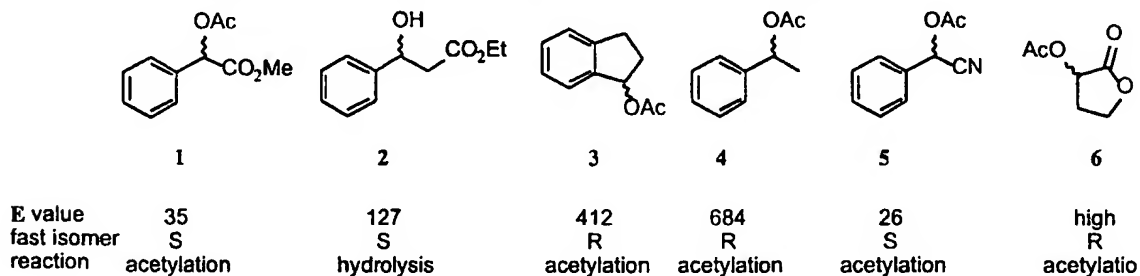
Example 2

pH-Dependent Assay for Hydrolase Activity

Bromothymol Blue (BTB) and Phenol Red were initially studied for use in this assay, as the pK_a values of these indicators are the closest to that of the phosphate buffer to be utilized and color transitions resulting therefrom show high contrast. Preliminary experiments showed that the concentration of indicator dye in the reaction had no effect on the reaction rate, suggesting that the indicator was not acting as an inhibitor of enzyme activity (data not shown). Control experiments using BSA as the protein source caused no change in indicator color and established that pH changes in solution were the result of enzyme catalyzed hydrolysis. Further tests of reaction solutions containing enzymes and indicators without substrates established that color changes in the solutions were not the result of buffer salts or the enzymes themselves. The basic principle governing this reaction is shown below:



In order to prove the concept of monitoring enantioselectivity using this method, we chose an array of enantiomeric substrates described in the literature to be efficiently resolved by well-known enzymes. A widely utilized lipase biocatalyst has been isolated from *Pseudomonas cepacia* ("PSL" available from Amano Pharmaceutical, Japan). This enzyme has been utilized for enantioselective hydrolysis as well as transesterifications in organic solvents. (Xie, Z.-F. *Tetrahedron: Asymmetry* **1991**, 2, 733.) The substrates shown below display enantioselectivities in the desired range (from high to very high).



The enantioselectivity (E) values are shown together with the fast-reacting isomer and the kind of reaction described in the literature. Compounds 1 through 6 shown above are ready to use in the hydrolytic assay. The substrates used were: methyl mandelate acetate (1), E = 35 for the acetylation of butyl ester (Ebert, et al. *Tetrahedron: Asymmetry* **1992**, 3, 903) and E = 18 for the methyl ester using the related AK lipase from Amano (Miyazawa, et al., *S. J. Chem. Soc. Perkin Trans. 1*, **1992**, 2253); ethyl 3-hydroxy-3-phenylpropionate (2), (Boaz, N. W. *J. Org. Chem.*, **1992**, 57, 4289); 1-indanol acetate (3) (Margolin, et al. *J. Am. Chem. Soc.*, **1991**, 113, 4693); 1-phenylethanol acetate (4) (Laumen, et al. *J. Chem. Soc. Chem. Commun.*, **1988**, 598; Laumen, et al. *J. Chem. Soc. Chem. Commun.*, **1988**, 1459; Seemayer, et al. *Tetrahedron Asymm.*, **1992**, 3, 827; Bianchi, et al. *J. Org. Chem.*, **1988**, 53, 5531; Terao, et al. *Chem. Pharm. Bull.*, **1989**, 37, 1653; Bianchi, et al. *Tetrahedron Asymm.*, **1993**, 4, 777; Keumi, et al. *Chem. Lett.*, **1991**, 1989; Gutman, et al. *Tetrahedron Asymm.*, **1993**, 4, 839); mandelonitrile acetate (5) (van Almsick, et al. *J. Chem. Soc. Chem. Commun.*, **1989**, 1391; Effenberger, et al. *Liebigs Ann. Chem.*, **1991**, 47; Inagaki, et al. *J. Am. Chem. Soc.*, **1991**, 113, 9360; Inagaki, et al. *J. Org. Chem.*, **1992**, 57, 5643); α -hydroxy- γ -butyrolactone

acetate (6) (Naoyuki, et al. *Eur. Patent App.* 1992, app # 91110749.8; Miyazawa, et al. *Eur. Patent App.* 1991, app # 90124577.9). In the case of mandelonitrile, only the R isomer was available and it was compared to the racemic mixture. The comparison is valid if the single isomer (R) is the slow reacting one so the racemic mixture will change color faster. Compounds 1, 3, 4, and 6 are only available as alcohols (both isomers) as well as (+)5 racemic mixture), and therefore must be acetylated prior to the assay. Since many of them are available as alcohols, the E values have been described for the transesterification reaction, not for hydrolysis.

The reactions were set up in a 96-well microplate and the total volume was 200 μ L split as follows: 100 μ L of PSL enzyme (1 mg, from a 10 mg/mL solution in buffer and spun off to avoid turbidity), 10 μ L of substrate solution (60 mg/mL for a final concentration of 3 mg/mL) and 90 μ L of 20 mM buffer solution pH = 7.20 containing 0.001% of indicator dye. The amount of PSL enzyme was 1 mg per well, which allowed a convenient time-window of no more than 48 h for the turnover of one of the isomers.

The concentration of the buffer employed was 20mM, weak enough to be saturated by the carboxylic acid being produced and drive the pH to the turnover point of the indicator. Five mM and 10 mM KPi buffers were also tested, and change color at a faster rate, but the sensitivity of the system was too high and minor pH shifts due to the background (after adding the enzyme or the substrate) led to inconsistent readings.

Ten microliters of single isomers 1-4 and 6 (or R-isomer 5 and racemic 5) solution were added, an amount representing (at 60 mg/mL) 0.6 mg per 200 μ L well (3 mg/mL final concentration). Since the molecular weight of the substrates is between 144 and 208, the molar concentration of the substrates ranges from 14-20 mM. This low concentration allowed for the use of very little pure enantiomer, so for example, 1 mL stock solution would last for 100 experiments.

As for the indicators used, Bromothymol Blue was the most extensively employed, although phenol red displayed good contrast, and two of the time points obtained are shown for comparison. **Figure 1** depicts the results obtained with both indicators and PSL enzyme. As can be seen in **Figure 1**, the enantioselectivity described above corresponds with the faster turnover (development of yellow color) of the preferred isomer in all cases studied. Since the hydrolysis proceeds slowly, the time for monitoring the progress of the reaction is large. While substrates 2, 3, 4 and 6 are hydrolyzed in 1-3 h, substrates 1 and 5 required longer reaction times. In this case, the same biocatalyst is used to evaluate a library of substrates, therefore the nature of these will dictate the time frame of the assay.

An advantage of the method is noted in the case of mandelonitrile acetate (5), with only one of the isomers available. Comparison of these results to those obtained using the racemic mixture yields the conclusion that the racemate turns color much faster than the R isomer. This is most likely due to the faster hydrolysis of the S isomer present in the racemate; thus, the skilled artisan is led to the conclusion that the enzyme is S-selective. It is not necessary to count on both isomers to run the screen, although a successful result may be more difficult to obtain in the absence of one of the isomers.

In the case of using Phenol Red as indicator, **Figure 1** shows essentially the same results. A control without enzyme or substrate is also shown. In this case the pH of the buffer is slightly higher (7.4) in order to obtain the red initial color. This could be the cause of the substrate S-1 being hydrolyzed apparently faster than in the bromothymol blue case. Thus, phenol red is a good choice if the reaction has to be carried out at slightly basic pH.

Example 3

Identification of Enantioselective Enzymes from an Enzyme Library

An application of the method is illustrated by the hierarchical screening of a ThermoCat[®] library of thermophilic esterases against α -hydroxy- γ -butyrolactone (6). This compound is a useful in the preparation of 4-substituted-2-hydroxy-butanoates and other optically and physiologically active compounds. The resolution can be visualized following either transesterification or hydrolysis of the corresponding acetate. The conclusions from the hydrolysis may be translated into the transesterification, at least in terms of enantioselectivity. However, the stability of the enzyme in organic solvent may jeopardize the resolution.

The commercial hydrolase kit from ThermoGen consists of 20 different hydrolases (E001 to E020) which all hydrolyze ester bonds and are organized in a 5x4 microplate array using BTB-containing 20mM KPi buffer at pH 7.2 and substrate at 3 mg/mL, as explained above. Total volume is 210 μ L, and every one of kit enzymes (10 units each) were dissolved in 1 mL of buffer, from which 200 μ L was added to the microplate wells (2 units per experiment). This first round of hierarchical screening involved all the enzymes in the kit was utilized for identification of enzymes that react with the racemic substrate such that the enzymes having the fastest reaction times would be selected. As such, the pure isomers were not required for screen against negatives.

Figure 2 depicts the process, and in this first round several enzymes were identified as potential candidates for resolution of the hydroxylactone: E004, E009, E011, E013, E015, E017b and E018b were selected for the enantioselectivity assay shown at the bottom of **Figure 2**. In this case, single isomers of the acetate were tested side by side using the same protocol as above. The control (substrate but no enzyme) and the PSL experiment are also shown in the same experiment. It is apparent that enzymes E004, E009, E011 and E013 are good candidates for further study, because a color change corresponding to the R isomer occurred in 20 minutes, as does PSL. On the other hand, E017b appears to react more slowly. E018b does not appear to be selective according to the observations during the first 3 hours of reaction. Thus, this method allows for the identification of potentially selective enzymes that should be studied further, and for identification of non-selective enzymes that should not be further considered.

Example 4

Identification of a DNA Molecule Encoding an Enzyme Having Ester-Hydrolyzing Activity

Provided herein is a method for isolating a DNA molecule encoding an enzyme having ester-hydrolyzing activity. First, a host cell is transformed with a DNA fragment encoding an enzyme having ester-hydrolyzing activity. Those host cells expressing the enzyme are then identified by selecting for expression of the ester-hydrolyzing enzyme in the host cell by detecting activity against an ester containing substrate in the presence of a pH indicator that provides a color change following a drop in the pH of the reaction mixture. The ester-hydrolyzing activity is detected in a pH-dependent reaction by preparing a crude lysate of the transformed cell, incubating a sample of the crude lysate in a buffer containing a pH-dependent indicator dye and a substrate to form a mixture, and monitoring the mixture for a color change.

The DNA molecule encoding the enzyme is then isolated from the host cell and the sequence encoding the hydrolase determined by DNA sequencing. It may be advantageous to subject the DNA fragment to multiple rounds of selection following enzymatic digestion in order to isolate smaller DNA fragments for analysis. Using this method, multiple host cell populations may be screened simultaneously, with increased speed and accuracy as compared to systems presently available.

Example 5

Identification of a DNA Molecule Encoding Mutated Ester-Hydrolyzing Enzyme

A method for isolating a DNA molecule encoding an enzyme having ester-hydrolyzing activity where the activity is altered as compared to the naturally-occurring enzyme is also provided. To identify such an enzyme, a host cell transformed with a fragment of a DNA encoding an enzyme having ester-hydrolyzing activity is exposed to conditions causing mutagenesis of the DNA molecule. For instance, the host cell is exposed to ethylmethane sulfanoate, *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine, or ultraviolet light. Those host cells expressing the enzyme are then identified by selecting for expression of the ester-hydrolyzing enzyme in the host cell by detecting activity against an ester containing substrate in the presence of a pH indicator that provides a color change following a drop in the pH of the reaction mixture. The ester-hydrolyzing activity is detected in a pH-dependent reaction by preparing a crude lysate of the transformed cell, incubating a sample of the crude lysate in a buffer containing a pH-dependent indicator dye and a substrate to form a mixture, and monitoring the mixture for a color change. Host cells demonstrating altered ester-hydrolyzing activity as compared to the wild-type enzyme are then selected. The DNA molecule encoding the enzyme is then isolated from the host cell. To determine the nature of the mutation, the portion of the DNA molecule encoding the enzyme is sequenced.

Example 6

Identification of Mutations Resulting in Expression of Ester-Hydrolyzing Enzymes Having Altered Activity

A method for isolating a DNA molecule encoding an enzyme having ester-hydrolyzing activity where the activity is altered as compared to the naturally-occurring ester-hydrolyzing enzyme is provided. A DNA molecule encoding an enzyme having ester-hydrolyzing activity is mutated by random or directed mutagenesis or to form a mutated DNA molecule. A host cell is then transformed with the mutated DNA molecule. Those host cells expressing the enzyme are then identified by selecting for expression of the ester-hydrolyzing enzyme in the host cell by detecting activity against an ester containing substrate in the presence of a pH indicator that provides for a color change following a drop in the pH of the reaction mixture. The ester-hydrolyzing activity is detected in a pH-dependent reaction by preparing a crude lysate of the transformed cell, incubating a sample of the crude lysate in a buffer containing a pH-dependent indicator dye and a substrate to form a mixture, and

monitoring the mixture for a color change. The DNA encoding the mutated ester-hydrolyzing enzyme is then isolated. Using this method, those mutations that confer an altered activity upon the enzyme are then identified.

While a preferred form of the invention has been shown in the drawings and described, since variations in the preferred form will be apparent to those skilled in the art, the invention should not be construed as limited to the specific form shown and described, but instead is as set forth in the claims.

CLAIMS

We claim:

1. A method for the visualization of hydrolase-catalyzed reactions using pH indicators, said method comprising the steps of, in combination:
 - a) combining an enzyme to be tested, a substrate to be tested, and an appropriate buffer in a reaction mixture;
 - b) combining into said reaction mixture a pH-sensitive indicator that is selected based on the predicted chemical reaction of said enzyme and said substrate; wherein said indicator will change the color of the reaction mixture in relation to progress of a chemical reaction between said enzyme and said substrate.
2. A method of claim 1 wherein said buffer is inorganic or organic.
3. A method of claim 2 wherein said inorganic buffer is a potassium phosphate, sodium phosphate or sodium bicarbonate buffer.
4. A method of claim 2 wherein said organic buffer is selected from the group consisting of (N,N-bis[2-hydroxyethyl]-2-aminomethanesulfonic acid (BES), Tris, citric acid, and N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES).
5. A method of claim 1 wherein said indicator is selected from the group consisting of bromophenol blue, neutral red, phenol red, brilliant yellow, cresol red, turmaric, metacresol purple, 2-(2,4-dinitrophenylazo)-1-naphthol-3,6-disulphonic acid, and 6,8-dinitro-2,4-(1H)quinalolinedione.
6. A method of claim 4 wherein said indicator is bromothymol blue or neutral red.
7. A method of claim 1 wherein said enzyme is selected from the group consisting of a lipase, protease and an esterase.
8. A method of claim 1 wherein said enzyme is chemically modified by addition of one or more compounds selected from the group consisting of phenyl glyoxal, EOC, EFA, NEM, dithiothreitol (DTT), PLP, phenylmethylsulfonyl fluoride (PMSF), and TNM.

9. A method of claim 1 wherein said method further comprises the addition of one or more chiral modifiers to the buffer such that the E of the reaction is altered.
10. A method of claim 9 wherein said chiral modifier is a chiral amine.
11. A method of claim 1 wherein said method further comprises the addition of one or more bile salts to the reaction mixture.
12. A method of claim 1 wherein said method further comprises the addition of one or more detergents to the reaction mixture.
13. A method of claim 12 wherein said detergent is Triton X-100.
14. A method of claim 1 wherein said enzyme has ester-hydrolyzing activity that is detected in a pH-dependent reaction comprising the steps of, in combination:
 - a) preparing a crude lysate of a cell transformed with a DNA molecule encoding an enzyme having ester-hydrolyzing enzyme under conditions wherein said enzyme is expressed in said cell;
 - b) incubating a sample of the crude lysate in a buffer containing a pH-dependent indicator dye and a substrate to form a mixture;
 - c) monitoring the mixture for a color change;wherein a color change of the mixture indicates the presence of an enzyme having ester-hydrolyzing activity.
15. A method for isolating a DNA molecule encoding an enzyme having ester-hydrolyzing activity comprising the steps of, in combination:
 - a) transforming a host cell with a DNA fragment comprising a nucleotide sequence encoding an enzyme having ester-hydrolyzing activity to generate a transformed host cell;
 - b) selecting for expression of the enzyme in the host cell by detecting ester-hydrolyzing activity by the method of claim 1;
 - c) isolating said DNA molecule from said host cell;whereby a DNA molecule comprising nucleotide sequence encoding an ester-hydrolyzing enzyme is obtained.

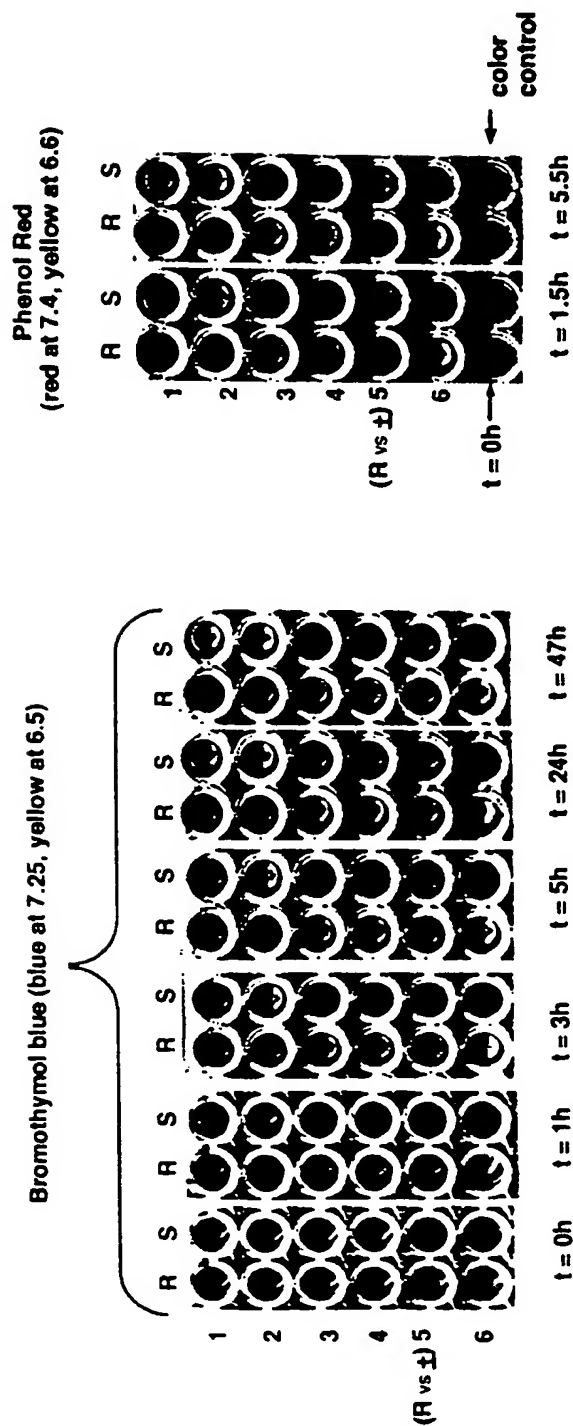
16. A DNA molecule isolated by the method of claim 15.
17. An enzyme encoded by the DNA molecule of claim 16.
18. A method for isolating a DNA molecule encoding an enzyme having ester-hydrolyzing activity where the activity is altered as compared to the naturally-occurring enzyme comprising the steps of, in combination:
 - a) exposing a host cell transformed with a DNA molecule encoding an enzyme having ester-hydrolyzing activity to conditions causing mutagenesis of said DNA molecule;
 - b) selecting for the expression of said enzyme by said host cell by detecting ester hydrolysis; and,
 - c) isolating said DNA molecule from said host cell;whereby a DNA molecule encoding an enzyme having altered activity over the naturally-occurring enzyme is obtained.
19. A DNA molecule isolated by the method of claim 18.
20. An enzyme encoded by the DNA molecule of claim 19.

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FIGURE 1

- Proof of concept: highly selective enzyme-substrate combinations: PSL and substrates 1-6.
Compare color development during the course of the hydrolysis.

E	notes	1	2	3	4	5	6
18 (S/R)	acetylation	<chem>CC(=O)OC(c1ccccc1)C(=O)OC</chem>	<chem>CCOC(=O)C(O)(c1ccccc1)CC</chem>	<chem>CCOC(=O)C1Cc2ccccc2C1</chem>	<chem>CC(=O)C(c1ccccc1)C</chem>	<chem>CC(=O)C(c1ccccc1)C#N</chem>	<chem>CC(=O)C1Cc2ccccc2C1=O</chem>
127 (S/R)							
412 (R/S)	acetylation						
684 (R/S)	acetylation						
26 (S/R)	acetylation						
high (R/S)							

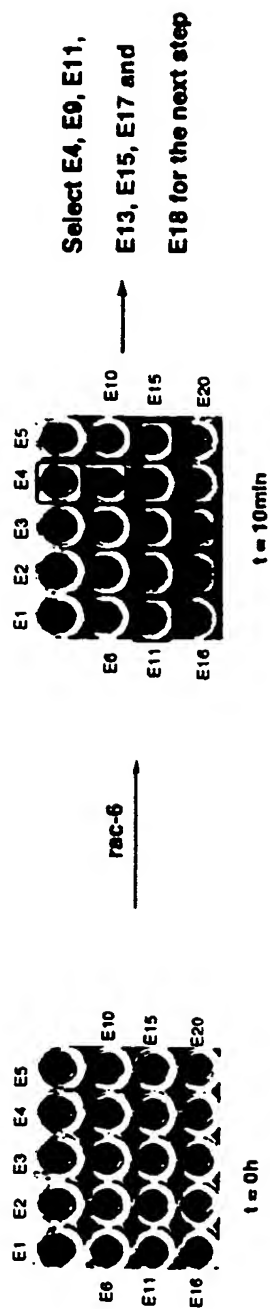


2/2

FIGURE 2

• Screening ThermoCat® library for the resolution of **6**. Quick identification of useful biocatalysts in two steps:

- Step 1: test library vs the racemic substrate:



- Step 2: test fastest positives from Step 1 vs. each enantiomer individually:

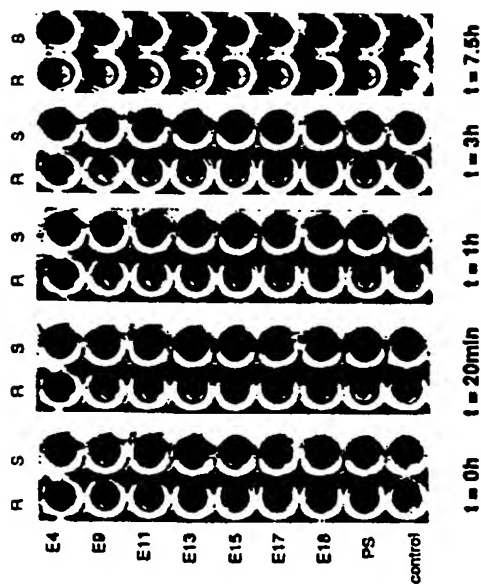


Fig 3.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12Q 1/34, 1/37, 1/44, C12N 15/12		A3	(11) International Publication Number: WO 00/01842 (43) International Publication Date: 13 January 2000 (13.01.00)
(21) International Application Number: PCT/US99/15400 (22) International Filing Date: 7 July 1999 (07.07.99) (30) Priority Data: 60/091,880 7 July 1998 (07.07.98) US 60/125,708 23 March 1999 (23.03.99) US (71) Applicant (for all designated States except US): THERMO-GEN, INC. [US/US]; 2225 W. Harrison Street, Chicago, IL 60612 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): MORIS-VARAS, Francisco [ES/US]; 651 S. Wells #201, Chicago, IL 60607-4500 (US). (74) Agent: HALLORAN, Patrick, J.; McDonnell, Bochnen, Hulbert & Berghoff, Suite 3200, 300 South Wacker Drive, Chicago, IL 60606 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 4 May 2000 (04.05.00)	
(54) Title: SCREENING OF HYDROLASE LIBRARIES FOR ENANTIOSELECTIVE ENZYMES			
(57) Abstract <p>The use of pH indicators to monitor enzyme-catalyzed reactions is described. The formation of acid following an enzyme-mediated reaction, such as hydrolysis, causes a drop in the pH that can be visualized by a change in the color of the indicator-containing solution. Preferred indicators are those showing a color transition within the operational pH range of the enzyme. Using the present system, the enantioselectivity of enzymes such as lipases and esterases can be estimated using single isomers under the same conditions and comparing the color turnover for each one. The method is also useful for application to the hierarchical screening of a library of enzymes.</p>			

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/15400

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/34 C12Q1/37 C12Q1/44 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 25058 A (THERMOGEN INC ;ALLEN LARRY (US); AIKENS JOHN (US); DEMIRJIAN DAVID) 17 July 1997 (1997-07-17) page 6, line 19 - line 26; claim 6; figure 1 ---	1-14
X	DATABASE WPI Section Ch, Week 198450 Derwent Publications Ltd., London, GB; Class B04, AN 1984-311650 XP002121270 & SU 1 091 065 A (GASTROENTEROL RES I), 7 May 1984 (1984-05-07) abstract --- -/--	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

2 November 1999

Date of mailing of the international search report

20.03.2000

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/15400

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US ROBERTS J ET AL: "A colorimetric assay of pancreatic lipase: rapid detection of lipase and colipase separated by gel filtration." retrieved from STN Database accession no. 85110320 XP002121271 abstract & LIPIDS, (1985 JAN) 20 (1) 42-5., ---</p>	1
P,X	<p>DATABASE CHEMABS [Online] CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US JANES, LANA E. ET AL: "Quantitative screening of hydrolase libraries using pH indicators: identifying active and enantioselective hydrolases" retrieved from STN Database accession no. 130:91947 XP002121272 abstract & CHEM.--EUR. J. (1998), 4(11), 2324-2331, ---</p>	1-14
X,P	<p>MORIS-VARAS F. ET AL.: "Monitoring enzymatic reactions using pH indicators: rapid screening and estimation of the enantioselectivity of hydrolase libraries." AMERICAN CHEMICAL SOCIETY: "BOOK OF ABSTRACTS OF THE 217TH ACS NATIONAL MEETING", 21 March 1999 (1999-03-21), XP002120172 abstract -----</p>	1-14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/15400

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
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see FURTHER INFORMATION sheet PCT/ISA/210
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because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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1-14

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

The DNA molecule and enzyme of claims 16, 17, 19 and 20 are not characterised by any technical features of the claimed products themselves and can therefor not be considered to clearly define the subject-matter for which protection is sought (Article 6, first sentence; Rule 6.3 (a) PCT). Moreover the description does not provide any technical support allowing the formulation of a search for the claimed entities. This represents a serious deficiency under Article 6, third sentence and Article 5 PCT. This renders it impossible to execute a meaningful search in the sense of Article 17(2)(a)(ii)PCT. The search was not and will not be covered for claims 16, 17, 18 ,19 and 20.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-14 completely.

Method for monitoring hydrolase-catalysed reactions using pH-indicators.

2. Claim : 15 completely.

Method for selecting a DNA molecule encoding an enzyme having ester-hydrolysing activity.

3. Claim : 18 completely.

Method for selecting a DNA molecule encoding mutated ester-hydrolysing enzymes having altered activity.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/15400

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9725058 A	17-07-1997	AU 6897496 A CA 2242864 A CN 1215336 A EP 0952847 A US 5969121 A	01-08-1997 17-07-1997 28-04-1999 03-11-1999 19-10-1999
SU 1091065 A	07-05-1984	NONE	

Form PCT/ISA/210 (patent family annex) (July 1992)



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁷ : C12Q 1/34, 1/37, 1/44, C12N 15/12</p>	<p>A3</p>	<p>(11) International Publication Number: WO 00/01842 (43) International Publication Date: 13 January 2000 (13.01.00)</p>
<p>(21) International Application Number: PCT/US99/15400 (22) International Filing Date: 7 July 1999 (07.07.99) (30) Priority Data: 60/091,880 7 July 1998 (07.07.98) US 60/125,708 23 March 1999 (23.03.99) US (71) Applicant (for all designated States except US): THERMO-GEN, INC. [US/US]; 2225 W. Harrison Street, Chicago, IL 60612 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): MORIS-VARAS, Francisco [ES/US]; 651 S. Wells #201, Chicago, IL 60607-4500 (US). (74) Agent: HALLORAN, Patrick, J.; McDonnell, Boehnen, Hulbert & Berghoff, Suite 3200, 300 South Wacker Drive, Chicago, IL 60606 (US).</p>	<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With a revised version of the international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 4 May 2000 (04.05.00)</p> <p>(88) Date of publication of the revised version of the international search report: 8 June 2000 (08.06.00)</p>	

(54) Title: SCREENING OF HYDROLASE LIBRARIES FOR ENANTIOSELECTIVE ENZYMES

(57) Abstract

The use of pH indicators to monitor enzyme-catalyzed reactions is described. The formation of acid following an enzyme-mediated reaction, such as hydrolysis, causes a drop in the pH that can be visualized by a change in the color of the indicator-containing solution. Preferred indicators are those showing a color transition within the operational pH range of the enzyme. Using the present system, the enantioselectivity of enzymes such as lipases and esterases can be estimated using single isomers under the same conditions and comparing the color turnover for each one. The method is also useful for application to the hierarchical screening of a library of enzymes.

*(Referred to in PCT Gazette No. 23/2000, Section II)

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REVISED
VERSION

INTERNATIONAL SEARCH REPORT

International Application No
PL 1/US 99/15400

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/34 C12Q1/37 C12Q1/44 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 25058 A (THERMOGEN INC ;ALLEN LARRY (US); AIKENS JOHN (US); DEMIRJIAN DAVID) 17 July 1997 (1997-07-17) page 6, line 19 - line 26; claim 6; figure 1 ---	1-14
X	DATABASE WPI Section Ch, Week 198450 Derwent Publications Ltd., London, GB; Class B04, AN 1984-311650 XP002121270 & SU 1 091 065 A (GASTROENTEROL RES I), 7 May 1984 (1984-05-07) abstract --- -/--	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"B" document member of the same patent family

Date of the actual completion of the international search

2 November 1999

Date of mailing of the international search report

13. 04. 2000

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INTERNATIONAL SEARCH REPORT

International Application No

PC/US 99/15400

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>DATABASE MEDLINE [Online] US NATIONAL LIBRARY OF MEDICINE (NLM), BETHESDA, MD, US ROBERTS J ET AL: "A colorimetric assay of pancreatic lipase: rapid detection of lipase and colipase separated by gel filtration." retrieved from STN Database accession no. 85110320 XP002121271 abstract & LIPIDS, (1985 JAN) 20 (1) 42-5., ---</p>	1
P,X	<p>DATABASE CHEMABS [Online] CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US JANES, LANA E. ET AL: "Quantitative screening of hydrolase libraries using pH indicators: identifying active and enantioselective hydrolases" retrieved from STN Database accession no. 130:91947 XP002121272 abstract & CHEM.--EUR. J. (1998), 4(11), 2324-2331, ---</p>	1-14
X,P	<p>MORIS-VARAS F. ET AL.: "Monitoring enzymatic reactions using pH indicators: rapid screening and estimation of the enantioselectivity of hydrolase libraries." AMERICAN CHEMICAL SOCIETY: "BOOK OF ABSTRACTS OF THE 217TH ACS NATIONAL MEETING", 21 March 1999 (1999-03-21), XP002120172 abstract -----</p>	1-14

INTERNATIONAL SEARCH REPORT

Int. application No.
PCT/US 99/15400

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-14

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

The DNA molecule and enzyme of claims 16, 17, 19 and 20 are not characterised by any technical features of the claimed products themselves and can therefor not be considered to clearly define the subject-matter for which protection is sought (Article 6, first sentence; Rule 6.3 (a) PCT). Moreover the description does not provide any technical support allowing the formulation of a search for the claimed entities. This represents a serious deficiency under Article 6, third sentence and Article 5 PCT. This renders it impossible to execute a meaningful search in the sense of Article 17(2)(a)(ii)PCT. The search was not and will not be covered for claims 16, 17, 18 ,19 and 20.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-14 completely.

Method for monitoring hydrolase-catalysed reactions using pH-indicators.

2. Claim : 15 completely.

Method for selecting a DNA molecule encoding an enzyme having ester-hydrolysing activity.

3. Claim : 18 completely.

Method for selecting a DNA molecule encoding mutated ester-hydrolysing enzymes having altered activity.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/15400

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9725058 A	17-07-1997	AU 6897496 A CA 2242864 A CN 1215336 A EP 0952847 A US 5969121 A	01-08-1997 17-07-1997 28-04-1999 03-11-1999 19-10-1999
SU 1091065 A	07-05-1984	NONE	